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The Effects of Transforming Growth Factor- β 2 on Dopaminergic Graft Survival

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Dopaminergic cell transplantation is a promising therapeutic approach for the treatment of Parkinson's disease, the potential of which is limited due to poor survival and low dopamine content within engrafted tissue. In this study, the ability of transforming growth factor- β 2 (TGF- β 2) to influence transplant survival was evaluated. Cell suspensions containing fetal rat ventral mesencephalon (VM) cells were incubated prior to surgery with vehicle (DPBS), varying concentrations of TGF- β 2 (5–1000 ng/ml), or a pan-specific antibody against TGF- β (1D11, 100 ng/ml). VM cell suspensions (200,000 cells) were unilaterally implanted into the striatum of adult Sprague-Dawley rats ($n = 5$ –11 animals/group). Following a 3-week survival period, small but viable VM grafts containing tyrosine hydroxylase-positive (TH+) neurons and fibers were present in all animals. Addition of TGF- β 2 resulted in a steep, bell-shaped dose–response curve with a significant effect on TH+/dopamine cell survival. At 50 ng/ml TGF- β 2, the number of surviving dopamine neurons was increased twofold compared with controls. Addition of TGF- β 2 or 1D11 did not significantly influence graft volume. Further studies, possibly in combination with other neurotrophic factors, need to be performed to obtain a greater understanding of the effects of TGF- β on dopamine neurons and fetal VM cell engraftment.

Key words: Transplantation; Parkinson's disease; Neurotrophic; Neuroprotection; TGF- β

INTRODUCTION

Transplantation of dopamine (DA)-containing fetal ventral mesencephalon (VM) cells into the striatum is a promising treatment for Parkinson's disease (5,11,15,20,34,47). Numerous preclinical and clinical studies demonstrated survival and engraftment of dopamine neurons resulting in a significant improvement in parkinsonian symptoms (4,5,16,20,32). However, the potential of this therapy is limited, in part, due to the poor survival and low content of dopamine neurons within the implant. General estimates of cell survival are on the order of 10% (7–9,17,39), and careful dissection of the fetal VM yields less than 5% dopamine neuron content within the cell suspension or tissue fragment used for implantation (10).

A variety of methods have been evaluated aimed at increasing graft survival and dopamine content. These include the addition of biologically active agents (such as cytoprotectants and neurotrophic factors) to transplant

tissue (2,13,18,19,36,37,40,44,51,54); altering the physical properties of the graft or host (e.g., lowering the body temperature of the graft recipient) (22); or optimizing the surgical procedure as in the micrografting approach described by Nikkhah et al. (39). In particular, the addition of trophic agents prior to, during, or after implant variably increased graft volume and/or dopamine cell survival (9).

Transforming growth factor- β (TGF- β) is part of a large family of cytokines with pleiotropic effects on animal and cell physiology, including wound healing, extracellular matrix regulation, modulation of inflammatory cell infiltration, and immune modulation (33). TGF- β exists as three isoforms in mammals (TGF- β 1, 2, and 3) with varying and often complex effects on neural tissues (14,24,42,52). In the central nervous system (CNS), TGF- β is a potent neurotrophic factor with direct influences on cell growth, development, and differentiation. In vitro, TGF- β 2 regulates the survival of DA neurons (26,41), and protects against toxin-induced degeneration

(23,25). Although there is considerable evidence that TGF- β exerts powerful neurotrophic and neuroprotective effects on DA neurons in culture, the effect of TGF- β on DA survival in vivo has not been investigated to date.

Based on these neurotrophic and neuroprotective properties, this study investigated whether TGF- β 2 could enhance dopaminergic graft survival. VM cell suspensions were preincubated with TGF- β 2 across a wide range of concentrations (5–1000 ng/ml), vehicle control, or 1D11, a pan-specific antibody to TGF- β , and then implanted into the nonlesioned rat striatum. Following a 3-week survival, the influence of pretreatments on graft volume and dopamine content was investigated.

MATERIALS AND METHODS

Experimental Animals

Adult male and female Sprague-Dawley rats (Taconic, Germantown, NY) were housed under 12:12-h light/dark cycle and provided food and water ad libitum. All procedures were carried out under an approved IACUC protocol and in accordance with guidelines for the use of animals in research (38).

Preparation of Fetal Ventral Mesencephalic Cell Suspension

The floor of the VM was isolated from E14 rat embryos (day of mating counted as day 0). The VMs were dissected in Ca^{2+} - and Mg^{2+} -free Dulbecco's phosphate buffered saline (DPBS) and incubated in 0.05% trypsin-EDTA for 10 min. The VM tissue was then washed in Hanks buffered salt solution (HBSS) containing 50 $\mu\text{g}/\text{ml}$ human recombinant DNase and dissociated by using a three-step trituration process. Tissue was passed through three precalibrated Pasteur pipettes with decreasingly smaller apertures (≈ 1 , 0.7, and 0.5 mm), resulting in a single cell suspension. VM cell suspensions were centrifuged at 1000 rpm for 5 min at room temperature (RT), resuspended in sterile Ca^{2+} - and Mg^{2+} -free DPBS, and stored overnight at 4°C prior to transplant. Cell viability was checked the next morning and averaged 93% using trypan blue dye. One to four hours prior to surgery, TGF- β 2 (5, 10, 50, 100, 500, or 1000 ng/ml), 1D11 (100 ng/ml, a pan-specific antibody to TGF- β), or vehicle alone (DPBS) was added to the cell suspension, and final cell density was adjusted to 100,000 viable cells/ μl using DPBS. The VM cell suspensions, containing the varying concentrations of TGF- β , vehicle, or 1D11 were then used for transplantation.

Test Materials

Recombinant human TGF- β 2 (100 mg/ml) was produced at Genzyme in Chinese hamster ovary cells and purified on cation exchange and reversed phase columns

to approximately 95%. Anti-TGF- β antibody, 1D11, was produced at Genzyme as a monoclonal IgG1 antibody in SP2/0 myeloma cells and purified on a protein G column to 95% as assessed by SDS-PAGE.

Transplantation

Animals were anesthetized with ketamine/xylazine (\pm diazepam) and the head mounted in a stereotaxic frame. Two deposits (1 μl each) of VM cell suspension were injected into the right striatum (coordinates: A/P: +1.0, M/L: -2.5 , D/V: -4.5 and -5.5 mm, from bregma) at a rate of 0.2 $\mu\text{l}/\text{min}$ through a 26-gauge needle attached to a Hamilton syringe (200,000 total cell dose). The needle was left in place for 10 min after the second injection, then slowly withdrawn. Animals ($n = 5$ –11/group) were assigned to one of eight groups based on pretreatment of cells with TGF- β 2, 1D11, or vehicle (saline) as described above. Animals were transplanted over the course of several different surgical days. A combination of experimental doses and vehicle treatments was used on each day to counterbalance any bias on graft results for a given surgical session.

Histology

At 20 days postimplantation, animals were deeply anesthetized and transcardially perfused with phosphate-buffered 2% paraformaldehyde/0.03% glutaraldehyde. The brains were removed and postfixed for 48 h. Serial coronal sections were cut on a vibratome or cryostat at a 50- μm thickness. Brains cut with a cryostat were protected in sucrose prior to freezing. Two series of free-floating sections (spaced 300 μm apart), spanning the rostro-caudal extent of the striatum, were set aside for staining. The first series was washed in 0.1 M potassium phosphate buffer saline (kPBS) and preincubated in 5% normal goat serum (NGS) and 0.25% Triton X-100 in kPBS for 3 h. Sections were then incubated overnight in kPB containing a polyclonal antibody to tyrosine hydroxylase (TH, 1:4000; Pel-Freez), 5% NGS, and 0.2% Triton X-100 at 4°C. The sections were rinsed in kPB and incubated for 1 h in a biotinylated goat anti-rabbit IgG (1:200 diluted in 0.1% BSA, 0.1% Triton X-100 in kPBS; Vector Labs). The tissue was rinsed in kPB and incubated in peroxidase-conjugated avidin-biotin complex (1:50 diluted in 0.1% BSA, 0.1% Triton X-100 in kPBS; Vector Labs) for 1 h at room temperature. Antibody binding was visualized by reaction of the sections with 3',3'-diaminobenzidine and hydrogen peroxide. Stained sections were mounted on gel-coated slides, dehydrated, and coverslipped. The second series of sections through each graft was mounted on gel-coated slides, stained for hematoxylin and eosin (H&E), dehydrated, and coverslipped.

Quantitative Graft Histology

Slides were read under blinded conditions for all quantitative analysis. The number of TH+ neurons present within the entire boundaries of the graft (including cells that effluxed into the corpus callosum and cortex) for each section was counted at 20 \times magnification under brightfield microscopy and recorded. The total number of TH+ cells/graft was estimated by multiplying cell counts by 6; every sixth section was stained and counted through the graft. Counts were corrected using Abercrombie's formula (1) (cell diameter = 40 μ m, section thickness = 50 μ m). Graft area was measured in TH+ stained tissue sections as margins were easily distinguished between transplanted cells and displaced host tissue. The graft outline was traced from digital images acquired using a SPOT camera interfaced with a computer. Area (mm²) was determined using the SCION Image program (ported from NIH Image for the Macintosh by Scion Corporation and available on the Internet at <http://www.scioncorp.com>). Graft volume (mm³) was then estimated as the sum of the graft areas multiplied by the distance between sections. The results for TH+ cell counts and graft volume were calculated as means \pm SEM. Each set of data was subjected to one-way analysis of variance (ANOVA) and a Fischer LSD post hoc test.

RESULTS

General Graft Histology

Small, but viable, grafts were present in all 52 implanted animals. Grafts typically were compact, rounded or elongate clusters of cells that displaced host striatal tissue (Fig. 1A). Grafted cells were often found extending dorsally along the injection tract with a small secondary deposit in the corpus callosum and deeper layers of the overlying cortex. By H&E staining, grafts were populated by large, rounded cells that were well integrated, having indistinct margins with the host brain (Fig. 1B). Scattered macrophages were present along the injection tract in some animals, but there was no evidence of vascular cuffing or small cell infiltration suggestive of graft rejection. Graft size was fairly uniform across experimental groups (Fig. 2) with an average volume of 0.679 ± 0.130 mm³ (\pm SEM). There was no significant effect of TGF- β 2 pretreatment on graft volume, $F(7, 44) = 0.689$, $p \leq 0.6805$.

TH Cell Survival

Densely stained TH+ cells and fibers were present to varying degrees within all grafts. TH+ cells were typically dispersed along the outer margins with TH+ fibers and isolated cell bodies extending through the interior (Fig. 1C, D). Across all groups, the number of TH+ neu-

rons was significantly different, $F(7, 44) = 3.0849$, $p < 0.0098$. Pretreatment with TGF- β 2 yielded a steep, bell-shaped dose-response curve in relation to graft TH+ cell content, with a maximal effect at 50 ng/ml (Fig. 3). Compared with the saline control, the number of TH+ cells in the 50 ng/ml TGF- β 2 group was increased roughly twofold (203 ± 32 vs. 390 ± 105 , $p < 0.0048$). The number of TH+ graft neurons in the 10 ng/ml TGF- β 2 group was increased (nonsignificantly) by 20% over saline control. All other TGF- β 2 treatment groups had mean TH+ cell counts that fell below control values, but none of these group comparisons reached significance. There was no effect of pretreatment of cells with anti-TGF- β antibody, 1D11, on TH+ cell counts. Across all engrafted animals, there was a significant, positive correlation between the number of TH+ cells and graft volume ($r = 0.547$, $p < 0.05$) independent of treatment group. However, TH cell density (TH count/graft volume) was significantly ($p < 0.0002$) increased in the 50 ng/ml group, consistent with a selective effect of TGF- β 2 on dopamine survival postimplant.

DISCUSSION

Neural transplantation, as a form of dopamine cell replacement therapy for Parkinson's disease, has yielded encouraging results in preclinical models as well as in a limited series of human cases (5,11,15,20,34,47). However, these early gains have been tempered by a growing perception that clinically meaningful engraftment on a large scale awaits significant improvements in transplantation technology. The two most notable technical shortcomings are relatively poor cell survival and low dopamine cell content. In response, a large effort has been generated in preclinical models investigating methods to improve suboptimal engraftment including trophic supplementation of grafted tissues and cells.

Within this context, the present study evaluated graft augmentation through the addition of TGF- β 2 to the VM cell suspension prior to transplant. TGF- β was not evaluated previously in this model despite considerable evidence that it has potent developmental, neurotrophic, and neuroprotective effects on midbrain dopamine neurons. Across a wide range of concentrations (5–1000 ng/ml), TGF- β 2 yielded a maximal survival effect at 50 ng/ml with roughly a twofold increase in cell numbers over vehicle-treated animals. This magnitude of effect is comparable to numerous other factors previously used to supplement grafted tissue (9). However, grafts in the present study were small with relatively low numbers of surviving TH+ cells. The effects of TGF- β 2 on dopaminergic survival should be interpreted with caution pending further study of the molecule on larger grafts, possibly in combination with 6-OHDA denervation of the host striatum (see below). Nevertheless, the effective

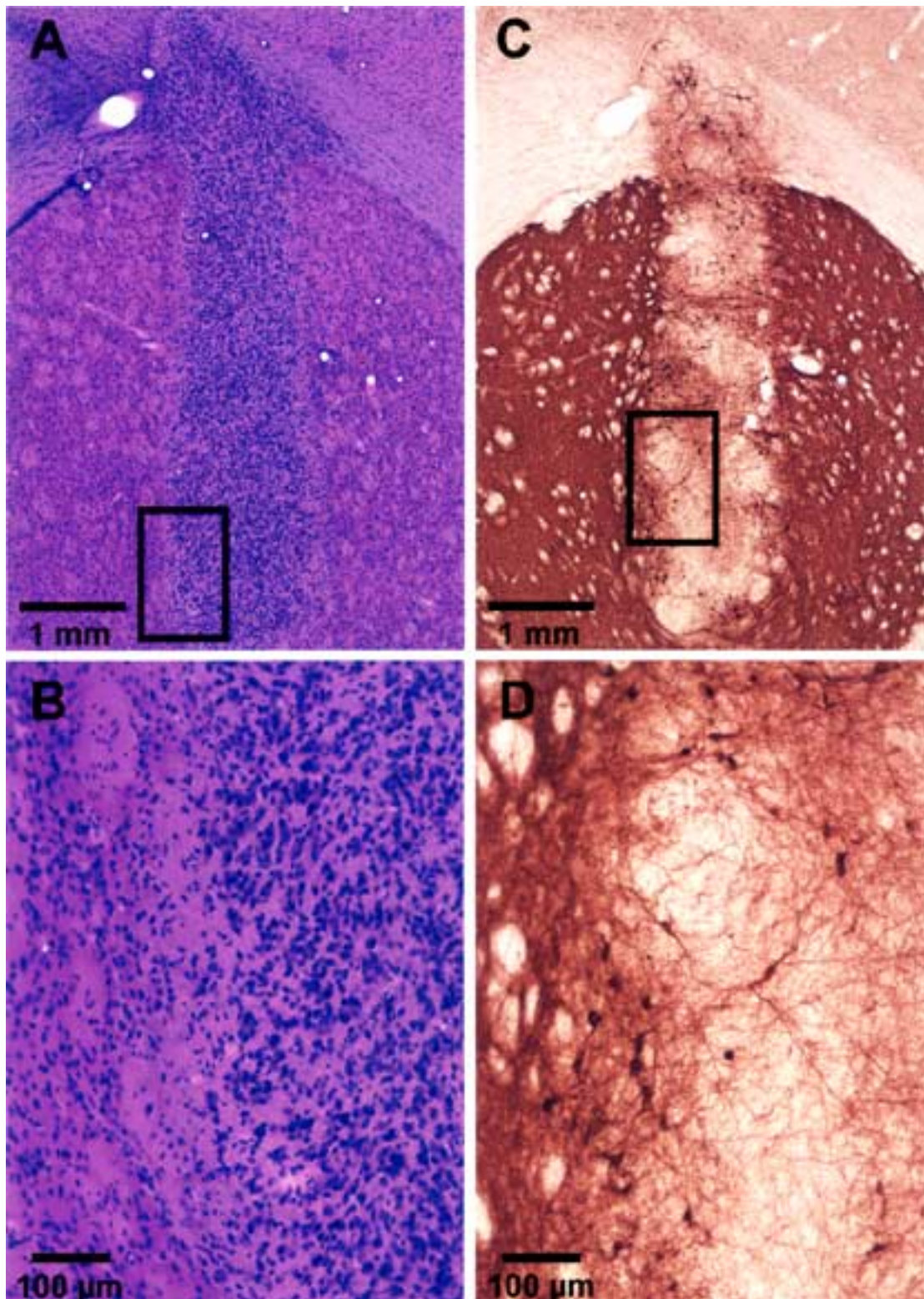


Figure 1. Photomicrographs of VM graft histology. H&E staining with views of a typical striatal VM graft (50 ng/ml TGF- β 2) at low (A) and higher magnification (B) showing good survival and integration within the host brain. TH staining of the same graft at low (C) and higher magnification (D) showing robust cell and fiber staining within the graft.

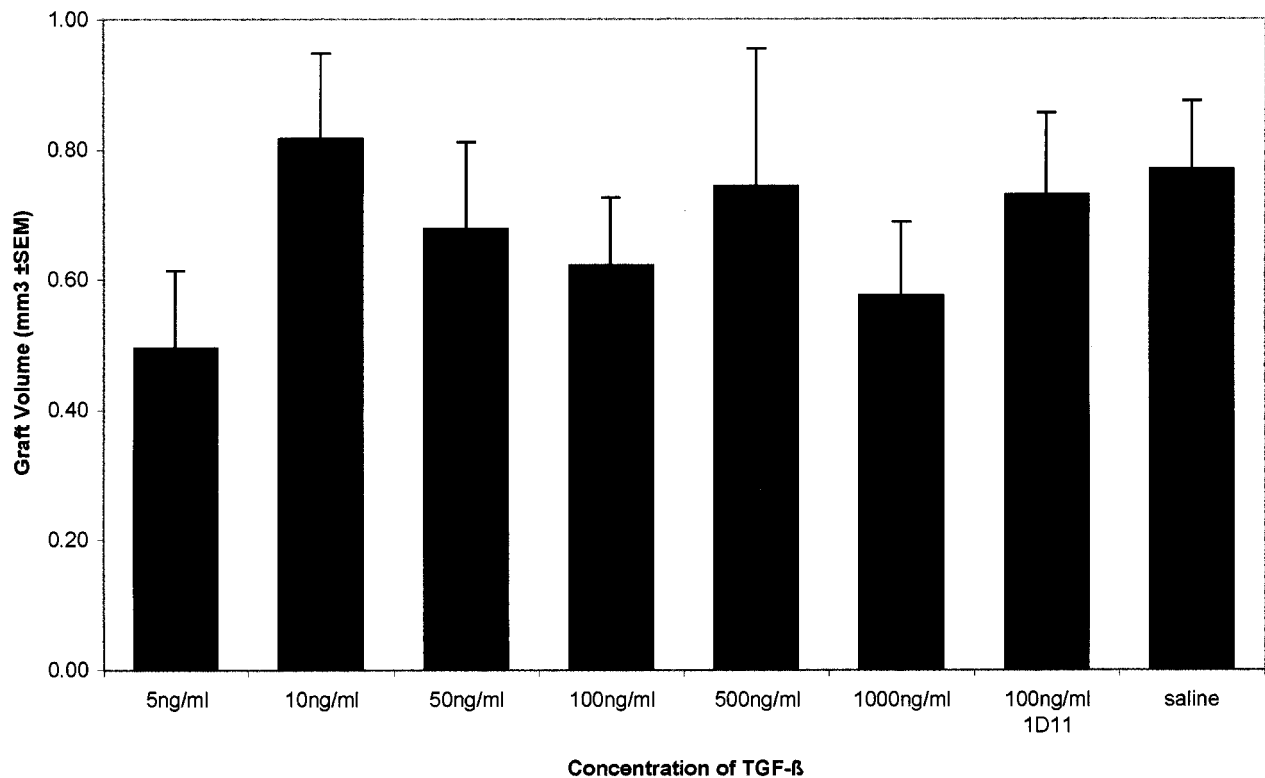


Figure 2. VM graft volume (mm³ ± SEM) across treatment groups. Graft size was fairly uniform and there were no significant differences in volume between groups.

concentration range of 10–50 ng/ml on TH survival in vivo for TGF- β 2 compares favorably with its in vitro activity. We found that concentrations of TGF- β 2 as low as 20 ng/ml significantly increased the survival of dopamine neurons within VM cultures (personal observation). Other investigators have also reported that TGF- β 2 in this concentration range increased dopamine cell survival in vitro (23,25,26,41).

At concentrations of TGF- β 2 greater than 50 ng/ml, the beneficial effects on TH cell survival in VM grafts were lost, suggesting that there may be an optimal concentration of TGF- β /receptor occupancy required for efficacy. This kind of dose–response curve is also seen in other aspects of TGF- β biology. For example, neutrophil and eosinophil chemotaxis show a similar bell-shaped response (35,43). The results highlight that cell survival in response to a “simple” additive may be quite complex (more may not be better) and underscores the need to do rigorous dose–response testing. Though no benefit was demonstrated, extremely high concentrations of TGF- β 2 (up to 1000 ng/ml) did not have a deleterious effect on graft survival or host brain. Likewise, addition of a pan-specific antibody to TGF- β (1D11) to the VM cell suspension had no apparent effect on subse-

quent engraftment. The concentration of 1D11 used in this study (100 ng/ml) is known to completely inhibit TGF- β activity using in vitro assays (48).

To date, multiple transplant approaches have been used, making it difficult to compare the efficacy of graft supplements on TH cell survival. This study used a typical cell “dose” of 200,000 cells/implant, and implantation was made into normal striatum as opposed to striatum denervated of dopaminergic innervation by 6-hydroxydopamine. It remains unclear if the denervated striatum is a more appropriate environment in which to evaluate graft survival (46,49). Lesion-induced changes in endogenous trophic expression within host striatum could influence the effect of TGF- β on subsequent TH cell survival within the VM grafts.

While the action of TGF- β on nervous tissue is often characterized as neurotrophic-like, it is clear that it has a more complex biological relationship with the nervous system (31). In particular, several studies by Kriegstein et al. (24,27,29) demonstrated that the main activity of TGF- β may be to regulate the relative potency of other neurotrophic factors. In vitro, the addition of TGF- β 2 (as well as TGF- β 3) synergistically increased trophic activity of CNTF, GDNF, and FGF-2 (28–30,53). In some

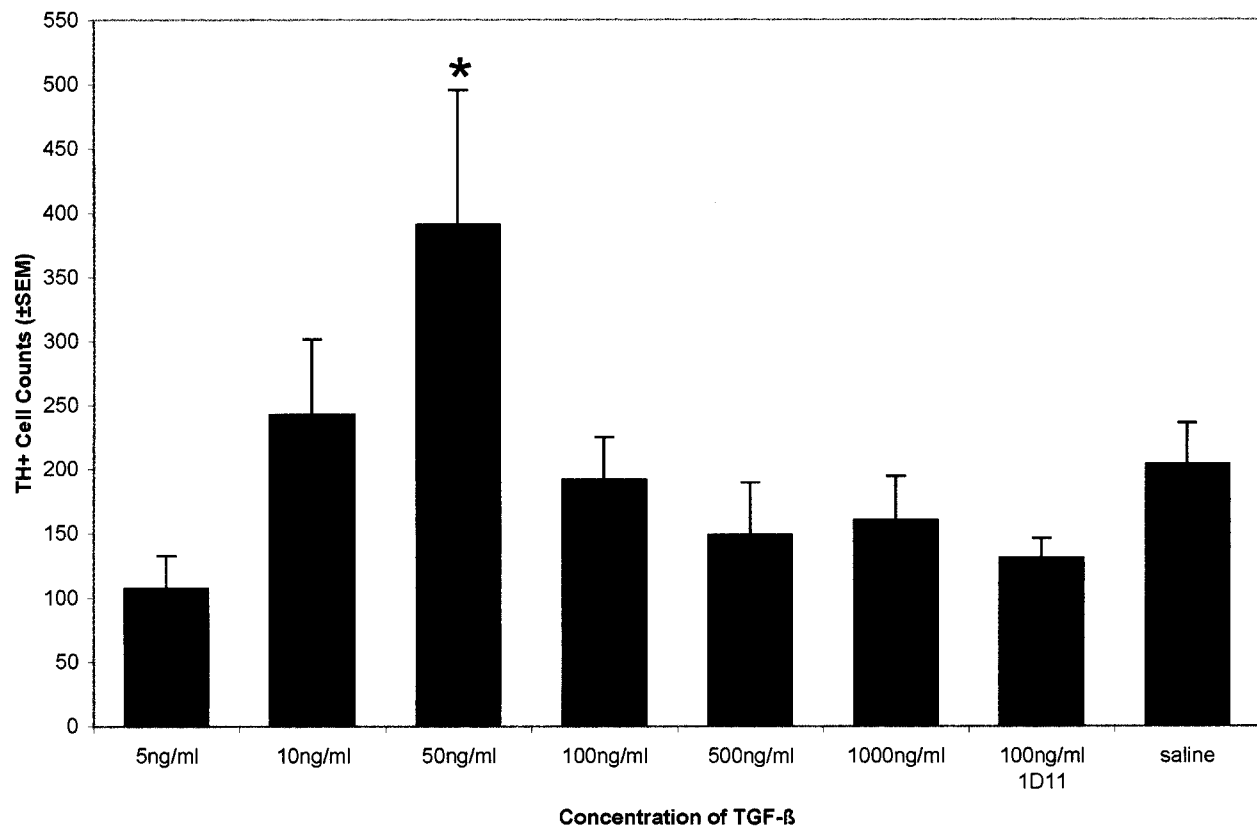


Figure 3. TH-positive cell content (mean \pm SEM) for VM grafts across treatment groups. Preincubation of VM cells with varying concentrations of TGF- β 2 resulted in a steep dose-response curve for TH cell counts. The only significant difference, $F(7, 44) = 3.0849$, $p < 0.0098$, was a twofold increase in survival in VM cells pretreated with 50 ng/ml TGF- β 2 compared with controls. The asterisk (*) denotes significance.

cases (e.g., chick ciliary ganglion cultures), TGF- β was found to have no survival-promoting effects unless it was co-incubated with another trophic factor (28). Subsequent transplant experiments should investigate whether incubating cells with cocktails of TGF- β 2 and GDNF or other factors will act synergistically to increase cell survival and engraftment.

The window of opportunity for manipulating grafts extends from initial dissection of donor tissue to chronic treatment of the host following implantation. Previous studies found that the majority of cell death occurs during mechanical dissociation of VM tissue or within the first few days following transplant (3,6,12,21,49,50,55). In this study, TGF- β 2 was added to the cell suspension a few hours prior to transplant. This was considered a simple, practical, clinically relevant way to provide trophic supplementation immediately prior to and during the initial engraftment period, which is a critical period of cell death for dopamine neurons. An earlier study by Zawada et al. (55) demonstrated increased numbers of TH+ cells and reduced apoptotic profiles at 24 h post-

transplant when tissues were incubated in growth factors for 2 h before transplant. In the present study, following 3-week survival, dopaminergic morphology had taken on a fairly mature appearance with elaboration of processes and the presence of large TH+ cells along the periphery of the VM graft or clustered within the interior. This length of survival is well past the critical period of cell death, and similar studies on trophic supplementation have used time points in the range of 2–8 weeks.

An exciting new advancement in transplant technology is the use of stem cells, particularly neural stem cells (45). The ability to culture and expand a cell source for transplantation offers significant advantages over the use of freshly derived tissue. Culturing of cells allows for in vitro manipulation, and already a tremendous effort has been directed at ways to induce and enrich for dopaminergic phenotype in neural stem cells. Here, too, the multiple activities of TGF- β , as a developmental factor, a trophic regulator, and possibly a differentiating agent, should be carefully assessed.

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